

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Brian S. HOOKER, Ziyu DAI
and Johnway GAO

Title: METHOD OF PRODUCING
HUMAN COAGULATION
FACTOR FROM WHOLE
PLANTS OR PLANT CELL
CULTURES

Prior Appl. No.: 09/080,010

Prior Appl. Filing Date: 5/14/98

Examiner: O. Zaghmout

Art Unit: 1649

CONTINUING PATENT APPLICATION
TRANSMITTAL LETTER

Assistant Commissioner for Patents
Box PATENT APPLICATION
Washington, D.C. 20231

Sir:

Transmitted herewith for filing under 37 C.F.R. § 1.53(b) is a:

☒ Continuation ☐ Division ☐ Continuation-In-Part (CIP)

of the above-identified copending prior application in which no patenting, abandonment, or termination of proceedings has occurred. Priority to the above-identified prior application is hereby claimed under 35 U.S.C. § 120 for this continuing application. The entire disclosure of the above-identified prior application is considered as being part of the disclosure of the accompanying continuing application and is hereby incorporated by reference therein.

Enclosed are:

- ☒ Specification, Claims, and Abstract (22 pages).
- ☒ Informal drawings (4 sheets, Figures 1-4).
- ☒ Unexecuted Declaration and Power of Attorney (4 pages).
- ☒ Preliminary Amendment

- ☐ Assignment of the invention to _____.
- ☐ Assignment Recordation Cover Sheet.
- ☐ Check in the amount of \$40.00 for Assignment recordation.
- ☐ Small Entity statement.
- ☐ Information Disclosure Statement.
- ☐ Form PTO-1449 with copies of ___ listed reference(s).

The filing fee is calculated below:

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Basic Fee							\$690.00		\$690.00
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Independents:	3	-	3	=	0	x	\$78.00	=	\$0.00
If any Multiple Dependent Claim(s) present:						+	\$260.00	=	\$0.00
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- ☐ A check in the amount of \$744.00 to cover the filing fee is enclosed.
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- ☐ The Assistant Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Assistant Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date

June 6, 2005

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COAGULATION FACTOR FROM WHOLE
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PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, Applicants respectfully request that the above-identified application be amended as follows:

In the Specification:

Please amend the specification as follows:

Page 1, after the title, please insert the following paragraph:

--This application is a continuation application of United States Serial No.
09/080,010 filed on May 14, 1998, pending.--

REMARKS

Applicant respectfully requests that the forthcoming amendment be made prior to examination of the present application.

Respectfully submitted,

Date June 6, 2000

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5
**METHOD OF PRODUCING HUMAN COAGULATION FACTOR VIII FROM WHOLE
PLANTS OR PLANT CELL CULTURES**

10 This invention was made with Government support under Contract DE-AC06 76RLO
1830 awarded by the U.S. Department of Energy. The Government has certain rights in the
invention.

FIELD OF THE INVENTION

15 The present invention relates generally to a method for producing a human coagulation
factor VIII from whole plants or plant cell culture. More specifically, the invention relates to a
method for producing a human coagulation factor VIII from a whole plant or plant cell culture
which results from a plant cell encoded to produce the human factor VIII. This invention is
directed to these associated embodiments in all respects. As used herein, the terms "human
factor VIII" and "human coagulation factor VIII" are interchangeable and refer to human like
20 proteins possessing human factor VIII sequence identity or human factor VIII-like procoagulant
or coagulant activity.

BACKGROUND OF THE INVENTION

25 Given the technological advances in recombinant DNA technology made over the past
decade it has become common practice to introduce new genetic material into plants cells, plants,
or plant tissue to establish new traits that enhance the value of the plant or plant tissue. The
present invention relates to a method for producing human blood coagulation factor VIII from a

whole plant or plant cell culture which result from a plant cell encoded to produce the human factor VIII.

Factor VIII is a highly specialized type of protein used primarily in the treatment of Hemophilia A as well as for other research and commercial applications. Factor VIII is a large glycoprotein that, when activated, functions in the blood coagulation cascade as a cofactor, along with calcium ions and phospholipid, in the factor IX mediated activation of factor X in the intrinsic coagulation pathway. It can be activated proteolytically by several coagulation enzymes, including thrombin.

Because of the difficulty in its production, plasma-derived factor VIII is in chronically short supply, therefore making this type of therapy highly cost prohibitive. In addition, resulting pharmaceutical products derived from blood plasma are highly impure, with a specific activity of 0.5 to 2 factor VIII units per milligram protein (one unit of factor VIII activity is by definition the activity present in one milliliter of normal plasma). Plasma-derived factor VIII purity is typically lower than 1% by weight (Wood et al. 1984. Nature 312:330). The high level of impurities result in a variety of serious complications including transmission of hepatitis A, B, and C, human parvovirus and human immunodeficiency virus (HIV) pathogens. To circumvent difficulties with viral pathogen transmission associated with human plasma-derived factor VIII and to lower product cost, factor VIII has been successfully expressed in a variety of mammalian cell culture systems. Initially, recombinant factor VIII was produced in baby hamster kidney (BHK) cell lines, using the calcium-phosphate coprecipitation method (Simonsen et al. 1983. Proc Natl Acad Sci USA 80:2495, Wigler et al. 1979. Proc Natl Acad Sci USA 76:1373) for integration of the 7kb protein encoding region (Wood et al. supra 1984, Capon et al. 1997. U.S. Patent No. 5618788). Active recombinant human factor VIII has also been produced in Chinese hamster ovary (CHO) cells (Kaufman et al. 1988. J Biol Chem 263:6352) and monkey COS-7

cells (Toole et al. 1984. *Nature* 312:342, Truett et al. 1985. *DNA* 4:333). However, production of factor VIII using these mammalian cell lines does not eliminate the potential for transmission of human pathogens. Consequently, these cell lines require additional quality assurance testing during biosafety trials to safeguard against virus transmission.

Other than the instant invention, production of factor VIII in recombinant hosts other than mammalian cells has not yet been successfully completed. Although factor VIII does not require γ -carboxylation of glutamic acid residues for activity, factor VIII does possess 25 separate glycosylation sites, which may preclude the use of prokaryotic host organisms for its production, especially if glycosylation is necessary for activity. In addition, the large (7.3 kb) protein coding region may preclude the use of many prokaryotic and lower eukaryotic hosts.

General Overview of Transgenic Plants for Foreign Protein Production

Transgenic plants can be used for the production of high value, medically important proteins, for example, monoclonal antibodies (Hiatt et al. 1989. *Nature* 342:76, During et al. 1990. *Plant Mol Biol* 15:281, Benvenuto et al. 1991. *Plant Mol Biol* 17:865, Firek et al. 1993. *Plant Mol Biol* 23:861, Magnuson et al. 1996. *Prot Expr Purif* 7:220), human growth hormone (Kay et al. 1987. *Science* 236:1299) and human serum albumin (Sijmons et al. 1990. *Bio/Technol* 8:217). Transformed cells synthesize, secrete, and accumulate functional antibodies including single (Benvenuto et al. supra 1991) and double (During et al. supra 1990, Hiatt et al. supra 1989) domain immunoglobulins. However, none of these authors reported the production of complex human blood coagulation factors from transgenic plants.

Unlike bacteria, plants perform many of the complex protein-processing steps required to produce mammalian proteins in an active form. Although most mammalian transgene products accumulate in plants at levels below 1% of soluble protein, Hiatt et al. (supra 1989) reported IgG antibody levels of up to 1.3% of soluble protein in tobacco. In addition, foreign protein levels of 14.4% soluble protein were reported in efforts to produce phytae, a digestive enzyme used in

livestock, in transgenic tobacco (Pen et al. 1993. Bio/Technol 11:811). Despite these efforts, there have been no reports of the successful production of any human blood coagulation factors from transgenic plants.

Whole plant and plant cell culture media are well-defined and relatively inexpensive to produce and work with when compared to mammalian cell culture media. Further, plant-based products, unlike mammalian-derived protein formulations, are generally assumed to be neither pathogenic nor oncogenic to humans (Cramer et al. 1996. Ann NY Acad Sci 792:62). Also, when compared to similar production in transgenic bacterial strains (Attaai et al. 1987. Biotechnol Bioeng 30:389), plant transformation methods showed greater stability of foreign gene expression, even without use of selection pressure (Gao et al. 1991. Plant Cell Reports 10:533).

Plants may be transformed to express foreign DNA using a variety of methods including *Agrobacterium* transformation (An 1986. Plant Physiol 81:86-91; Hoekema et al. 1985. Plant Mol Biol 5:8589), microprojectile bombardment (Klein TM, et al., Gene Transfer by Particle Bombardment, Plant Tissue Culture Manual, D1, p. 112, 1991, Kluwer Academic Publishers), pollen transformation (Saunders et al. 1997. U.S. Patent 5629183), chemical mediated uptake by protoplasts (Krens et al. 1982. Nature 296:72-74), and electroporation (Langridge et al. 1985. Plant Cell Reports 4:355-359).

Agrobacterium transformation

A. tumefaciens is the etiological agent of crown gall, a disease of a wide range of dicotyledons and gymnosperms (DeCleene et al. 1976. Bot Rev 42:389). Virulent strains of *A. tumefaciens* contain large, tumor-inducing (Ti) plasmids (at about 200 kb). When wounded plants or plant tissues are cocultivated with such *Agrobacterium* strains, a portion of the Ti plasmid, called T-DNA, is transferred to and integrated into the nuclear genome of the infected plant cells (Hermalsteens et al. 1980. Nature 287:654, Lichtenstein et al. 1987. Genet Eng 6:104). The T-DNA encoded genes are transcribed and translated in the plant tissues, resulting in auxin,

cytokinin and opine synthesis. The elevated auxin and cytokinin levels cause rapid plant cell proliferation, resulting in gall formation (Gheysen et al. 1985. DNA flux across genetic barriers: the crown gall phenomenon, In: Genetic Flux in Plants, Hohn B, et al., Eds., Springer, Wein, pp. 11-49).

To exploit Ti plasmids for genetic transformation, the native T-DNA sequence, responsible for gall formation, can be replaced with foreign DNA, including selectable markers as well as the gene of interest. The only components required for DNA transfer in the T-DNA region are the left and right border sequences (Zambryski et al. 1982. *J Mol Appl Genet* 1:361). However, since Ti plasmids are difficult to manipulate directly in vitro due to their large size, simplified systems have been developed. The most advanced method utilizes a binary vector system in which the binary vector contains the minimum elements required *in cis* (An supra 1986, An. 1987. *Meth Enzy* 153:292). Other functions necessary for the gene transfer mechanism are donated from a separate helper Ti plasmid. The binary vector may be directly manipulated in an *E. coli* host and transferred to *A. tumefaciens* containing the helper Ti plasmid, through biparental or triparental mating. Finally, the T-DNA region is integrated into the plant nuclear genome by cocultivation of plant material with transformed *A. tumefaciens*. Representative tissues that have been transformed using an Agrobacterium method include tobacco (Barton et al. 1983. *Cell* 32:1033), tomato (Fillatti et al. 1987. *Bio/Technol* 5:726), sunflower (Everett et al. 1987. *Bio/Technol* 5:1201), cotton (Umbeck et al. 1987. *Bio/Technol* 5:263), rapeseed (Pua et al. 1987. *Bio/Technol* 5:815), potato (Facciotti et al. 1985. *Bio/Technol* 3:241), poplar (Pythoud et al. 1987. *Bio/Technol* 5:1323) and soybean (Hinchee et al. 1988. *Bio/Technol* 6:915).

Microprojectile bombardment

In this method, gold or tungsten DNA-coated particles are accelerated towards target plant cells (Klein et al. 1987. *Nature* 327:70). This technique has been used to obtain stably transformed cultures of maize and tobacco (Klein et al. 1988. *Bio/Technol* 6:559) as well as

transient expression in onion. A comprehensive summary of microprojectile bombardment is given in Klein TM, et al., *Gene Transfer by Particle Bombardment*, Plant Tissue Culture Manual, D1, p. 112, 1991, Kluwer Academic Publishers.

5 Pollen transformation

In pollen transformation techniques, plant germplasm is transformed with foreign DNA by introducing the DNA into pollen grains by a technique such as electroporation (Mishra et al. 1987. *Plant Sci* 52:135), mating ova of the desired plant line with the transformed pollen, and selecting for the transformed germplasm. The germinating pollen, resulting seed, and the progeny can each be screened for expression of the foreign gene. The transformed pollen can be used as a vector for introducing the foreign DNA into plant lines of similar or dissimilar origin, including both monocots and dicots. To date, pollen collected from tobacco and corn plants has been stably transformed via electroporation (Saunders et al. supra 1997). In addition, tobacco plants have been stably transformed to produce β -glucuronidase by mating ova with transformed tobacco pollen.

Chemical mediated uptake by protoplasts

DNA uptake via chemical stimulation was developed for the direct transformation of both monocot and dicot protoplasts (Krens et al. supra 1982). In this method, the plant cell wall is first degraded enzymatically to form protoplasts, using standard techniques. The protoplasts and vector are then incubated in the presence of polyethylene glycol, which facilitates transformation via direct insertion. Either a direct gene transfer vector or a Ti plasmid may be used in transformation. Since protoplast transformation can facilitate measurable gene expression within 24 to 48 hours, this method has been used widely by many researchers (Pröls et al. 1988. *Plant Cell Reports* 7:221, Töpfer et al. 1988. *Plant Cell Reports* 7:225).

Electroporation

Introduction of DNA into plant protoplasts by treatment of the protoplasts with an electric pulse in the presence of the appropriate DNA is a process called electroporation (Fromm et al. 1985. Proc Natl Acad Sci USA 82:5824). Supercoiled or circular plasmid DNA is added to a suspension of protoplasts. The solution is mixed and subjected to a pulse of approximately 400 V/cm at room temperature for less than 10 to 100 μ sec. A reversible physical breakdown of the membrane occurs to permit DNA uptake into the protoplasts. The success of the electroporation method is dependent, in part, on optimizing parameters relative to the membrane, the DNA and the electric field. Evidence for the success of transformation after electroporation has been measured by incorporation of radioactively labeled DNA (Tsong et al. 1985. Biblio Haematol 51:108), transient gene expression (Potter et al. Proc Natl Acad Sci USA 1984. 81:7161, Smithies et al. 1985. Nature 317:230), and the formation of stable transformants (Riggs et al. 1986. Proc Natl Acad Sci USA 83:5602, Stopper et al. 1985. Z Naturforsch 40:929). Due to rapid performance associated with electroporation, this technique is often applied for quick isolation and characterization of plant promoters and cis-acting elements (An et al. 1993, Techniques for isolating and characterizing plant transcription promoters, enhancers, and promoters. In: Methods in Plant Molecular Biology and Biotechnology, Glick BR, et al., Eds., CRC Press, Boca Raton, pp. 155-165).

Plant (material) regeneration methods

After transformation of plant tissues, plants must be regenerated for characterization of stable transformants, selection, breeding and segregation analysis, and foreign protein (blood coagulation factor) production. Plants may be regenerated from a variety of tissues including callus culture, protoplasts, and *A. tumefaciens* tissues (i.e., explants or calli). Regeneration from callus tissue has been demonstrated in monocots, such as corn, rice, barley, wheat and rye and dicots, such as sunflower, soybean, cotton, rapeseed and tobacco.

Regeneration of plants from protoplasts is particularly useful for tissues transformed via direct gene transfer methods including electroporation, PEG-mediated transformation, or microparticle bombardment. Regeneration of plants from protoplasts has been demonstrated for rice (Abdulah et al. 1987. Bio/Technol 4:1987), tobacco (Potrykus et al. 1985. Mol Gen Genet 199:169), rapeseed (Kansha et al. 1986. Plant Cell Reports 5:101), and potato (Tavazza et al. 1986. Plant Cell Reports 5:243), among others. Regeneration of plants from tissue transformed with *A. tumefaciens* has been demonstrated for several species of plants including tobacco (Horsch et al. 1985. Science 225:1229, Herrera-Estrella et al. 1983. Nature 303:209), sunflower (Everett et al. supra 1987), tomato (Fillatti et al. supra 1987), rapeseed (Pua et al. supra 1987), and cotton (Umbeck et al. supra 1987), among others.

SUMMARY OF THE INVENTION

The production of factor VIII in transgenic plant systems has the potential to significantly reduce the cost and safety risks associated with current production methods for this product. For example, direct production costs for whole plant processes at equal protein production rates appear to be two to four orders-of-magnitude lower than comparable mammalian cell processes. As plant expression levels increase, this type of production becomes even more capital cost-effective. Additionally, economic analysis indicates that regulatory costs associated with plant cell culture may reduce by as much as \$70,000 per batch as compared to analogous mammalian cell processes because plants are regarded as free of human viral pathogens (Crawford. 1995. Therapeutic protein production using plant cell culture. Report by Lasure and Crawford, Inc., Seattle, Washington).

It is, therefore, an object of the present invention to provide whole plant and plant cell culture derived human coagulation factor VIII at lower cost when compared to mammalian host cell systems.

It is another object of the present invention to use *Nicotiana tabacum* (tobacco) and other plant species including those representing monocotyledonous and dicotyledonous plants to produce factor VIII.

According to the present invention, the production of human coagulation factor VIII is achieved in whole plants or plant cell culture wherein the human coagulation factor VIII is produced by the methods disclosed resulting in human factor VIII coagulant antigen (FVIII:ag) appearing to be correctly processed *in planta*.

Modifying encoding sequences and subcloning into a plant expression vector are done using standard molecular cloning procedures (Ausubel et al. 1992. Current protocols in molecular biology. Wiley, New York) and splicing PCR techniques (Marks et al. 1992. J Biol Chem 267:16007).

The success of the present invention is evidenced by:

(a) Immunological cross-reactivity of antibodies raised against plasma-derived factor VIII proteins with clone-derived factor VIII proteins as validated by enzyme-linked immunosorbent (dot blot) assay.

(b) Comparable relative protein size between the clone-derived factor VIII proteins and the plasma-derived factor VIII proteins as validated using Western blot immunoassay.

Thus, the present invention is based upon the successful use of plant molecular biology methods to produce human factor VIII *in planta*.

The subject matter of the present invention is particularly pointed out and distinctly claimed in the concluding portion of this specification. However, both the organization and method of operation, together with further advantages and objects thereof, may best be understood by reference to the following description taken in connection with accompanying drawings wherein like reference characters refer to like elements.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts schematically the construction of pSP64-FVIIIc, a vector used to obtain the encoding sequence for pre-coagulation factor VIII for cloning into the plant expression vector pGA748. Pre-coagulation factor VIII cDNA encoding the 2332 amino acid mature protein plus the 19 amino acid native signal was inserted into the pSP64 plasmid at the Sal I site using a Sal I adapter, resulting in the new 10200 bp pSP64-FVIIIc plasmid. This plasmid carried the ampicillin resistance gene for positive transformant selection in *E. coli*.

FIG. 2 depicts schematically the construction of pZD201, the plant expression vector carrying the pre-coagulation factor VIII encoding region. The full length pre-coagulation factor VIII cDNA was excised with Sal I restriction enzyme and sequentially ligated into the compatible restriction enzyme site Xho I located between the CaMV 35S promoter and T7-T5 transcript terminator of binary vector pGA748, forming the 18800 bp plasmid pZD201. This plasmid carried the tetracycline resistance gene for positive transformant selection in *E. coli* and *Agrobacterium tumefaciens* as well as the kanamycin resistance gene for selection of whole plant and plant cell culture positive transformants.

FIG. 3 shows the result of dot blot immunoassay for T0 Factor VIII plant transformants. Positive control plasma-derived factor VIII standards (American Diagnostica, Greenwich, CT) are shown as S1 and S2. Negative control leaf protein extracted from untransformed *Nicotiana tabacum* cv. SR1 is shown as SR. Positive plant primary transformants (leaf protein extracts) are shown as 1004-3, 1006-2 and 1006-3.

FIG. 4 shows protein gel bands resulting from Western blot immunoassay completed on protein extracts from several plant transformants as compared to a non-transformed control culture and plasma-derived factor VIII standard (American Diagnostica, Greenwich, CT). Lane

1 shows the plasma-derived factor VIII (FVIIIc) standard; lane 2 shows total protein extracts from leaf explants taken from an untransformed *N. tabacum* cv. SR1 control; lanes 3 through 9 show total protein extracts from leaf explants taken from T1 plant lines derived from primary transformant tobacco plants.

DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

The present invention is a method for production of human coagulation factor VIII using whole plants as well as plant cell suspensions transformed with appropriately constructed vector plasmids. More specifically, the method of the present invention is stable expression of human coagulation factor VIII:ag, that exhibits immunological cross-reactivity with antibodies to human factor VIII. It is contemplated that virtually any plant from the plant kingdom may be utilized. Specific types of plants that are amenable to the transformation steps disclosed herein include, but are not limited to monocotyledonous and dicotyledonous plants. Example species include but are not limited to *Nicotiana tabacum* (tobacco), *Solanum tuberosum* (potato), *Glycine max* (soybean), and *Zea mays* (corn).

The method of the present invention for producing a human coagulation factor VIII from plant cells, comprises the steps of:

- (a) introducing an encoding sequence for production of human coagulation factor VIII into a plant expression vector in the plant cells;
- (b) obtaining a positive transformant of the plant cells, which carries genetic material encoding the production of the human coagulation factor VIII;
- (c) cultivating the positive transformant; and
- (d) obtaining the human coagulation factor VIII.

The step (b) may be quite simple (such as purchasing transformants) or relatively more complex, resulting from actual transformation by well known methods, for example direct particle bombardment as described in Gene Transfer by Particle Bombardment, Klein TM, Knowlton S, Arentzen R, Plant Tissue Culture Manual, D1, pp 1-12, 1991, Kluwer Academic Publishers, or by Agrobacterium mediated transformation as described in Hoekema et al. (supra 5 1985), and further described herein.

The step (c) of cultivating involves either whole plant cultivating or tissue cultivating by any of several well known cultivating methods.

The step (d) of obtaining the factor VIII is by well known separation and purification steps, for example protein precipitation, ultrafiltration, affinity chromatography, and/or electrophoresis.

An agrobacterium mediated transformation method of the present invention comprises the steps of:

- (a) modifying the coagulation factor VIII encoding sequence for subcloning into a plant expression vector,
- (b) subcloning the encoding sequence into the plant expression vector;
- (c) transferring the plant expression vector to agrobacterium;
- (d) co-cultivating a portion of the transgenic plant cells (suspension culture or leaf disks) with the agrobacterium;
- (e) selecting positive transformants from the co-cultivated culture on an antibiotic selective media;
- (f) permitting growth of the transgenic plant cells in whole plants or suspensions; and
- (g) extracting a liquid containing the human coagulation factor VIII.

Modifying encoding sequences and subcloning into a plant expression vector are accomplished using standard molecular cloning procedures (Ausubel et al. supra 1992) and splicing PCR techniques (Marks et al. supra 1992). More specifically, modifying encoding sequences, comprises the steps of:

- (a) adding a transcription promoter to the upstream or 5' end of the factor VIII encoding sequence; and
- (b) adding a transcription terminator to the downstream or 3' end of the said encoding sequence. The transcription promoter and the transcription terminator are regulatory elements.

Further, an additional regulatory element encoding a signal peptide may be added between the transcription promoter and the 5' end of the encoding sequence in order to relegate the product human growth factor to a specific cellular organelle. In addition, other regulatory elements may be added either between the promoter and the additional regulatory element encoding the signal peptide or at the 3' end of the encoding sequence to obtain greater mRNA stability between transcription and translation events.

Transferring the plant expression vector into the agrobacterium is completed using the freeze-thaw method (An supra 1987). For monocotyledonous species, super-binary vectors, such as pTOK233 and pSB131, are used to achieve high transformation frequency (Ishida et al. 1996. Nature Biotechnol 14:745). Remaining cocultivation, selection, growth, and extraction steps (d through g) have been described by Magnuson et al. (supra 1996), and are well known in the art of plant molecular biology.

At least one genetic regulatory element may be included in the sequence encoding the transcription of human coagulation factor VIII. Regulatory elements include transcription promoters or enhancers that increase the frequency of transcription events, leader sequences that increase the stability of mRNA prior to translation, and signal peptides that target proteins to

specific organelles for posttranslational modifications and accumulation. Examples of transcription enhancers include but are not limited to the octapine synthase enhancer, a 16 bp palindrome (ACGTAAGCGCTTACGT) (Ellis et al. 1987. EMBO J 6:3203) and the B-domain of the cauliflower mosaic virus 35S promoter (Kay et al. supra 1987). An example of a leader sequence includes but is not limited to alfalfa mosaic virus RNA4 leader sequence (Jobling et al. 1987. Nature 325:622). Examples of signal peptides include but are not limited to the tobacco PR-S signal peptide (Cornelissen et al. 1986. Nature 321:531) and the phytohemagglutinin signal peptide (Hunt et al. 1991. Plant Physiol 96:18).

Example 1

The *Escherichia coli* plasmid pSP64-FVIII (ATCC No. 39812) containing the gene encoding the full-length polypeptide of factor VIII cDNA, derived from human fetal liver, was obtained from ATCC (FIG. 1). Coagulation factor VIII is the 2332 amino acid protein which contains a heavy chain, B-domain, and light chain. The 7.2kb pre-coagulation Factor VIII cDNA encodes the native signal peptide (the first 19 amino acid at the N-terminus) as well as the mature protein.

The full length pre-coagulation factor VIII cDNA was excised with Sal I restriction enzyme and sequentially ligated into the compatible restrict enzyme site Xho I located between the CaMV 35S promoter and T7 transcript terminator of binary vector pGA748, forming the 19 kb plasmid pZD201 (FIG. 2). This plasmid was directly transferred into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method (An supra 1987). The transferred plasmid was introduced into tobacco whole plants (by leaf disks) and calli (by suspension culture) by co-cultivation with agrobacterium thereby producing transformants. Over 200 specific samples of transformants were taken from the co-cultivation and separately placed on kanamycin selective

media. After obtaining positive transformants via kanamycin resistance screening, mature tobacco plants and calli were assayed for the presence of the human coagulation factor VIII. Preliminary protein immunoblotting (dot blot assays-FIG. 3) completed using extractable leaf protein showed the presence of coagulation factor VIII antigen in the leaf tissues of T0 whole plant transformants. As seen in FIG. 3, strong factor VIII:ag expression characteristics were seen in plants 1004-3, 1006-2 and 1006-3. Positive control factor VIII standards (American Diagnostica, Greenwich, CT) are shown as S1 and S2 whereas negative control leaf protein derived from untransformed *N. tabacum* cv. SR1 is shown as SR. After completion of the dot-blot immunoassay, T0 plants were self-pollinated, resulting in T1 seedstock. T1 seeds were subsequently germinated on kanamycin selective media and mature plants were grown in a controlled environment. Western immunoblot assays shown on FIG. 4, completed on leaf protein extracts of T1 plants regenerated from various T0 plant lines, indicate the presence of immunoreactive bands which appear to be comparable in size to those of plasma-derived factor VIII (American Diagnostica, Greenwich, CT). The predominant band appears at approximately 240 kD. Additional faint bands from plasma derived factor VIII and plant transformants appear to correspond to factor VIII heavy chain at 90-200 kD and light chain at approximately 80 kD. The appearance of comparable immunoreactive bands between plant-derived and plasma-derived human factor VIII suggest that plant-derived factor VIII undergoes correct, human-like post-translational modifications. Sheep anti-human factor VIII:C polyclonal antibody (Haematologic Technologies, Inc., Essex Jct., VT) and sheep anti-human factor VIII polyclonal antibody AB787 (Chemicon International, Inc., Temecula, CA) were mixed and used for both the Western blot and dot blot immunoassays.

CLOSURE

While a preferred embodiment of the present invention has been shown and described, it will be apparent to those skilled in the art that many changes and modifications may be made
5 without departing from the invention in its broader aspects. The appended claims are therefore intended to cover all such changes and modifications as fall within the true spirit and scope of the invention.

Claims

We claim:

1. A method of producing human coagulation factor VIII, comprising:
 - (a) subcloning an encoding sequence into a plant expression vector and obtaining a subcloned plant expression vector;
 - b) transferring the subcloned plant expression vector into a plurality of plant cells;
 - (c) selecting a plurality of positive transformants from the plurality of plant cells on an antibiotic selective media;
 - (d) growing the plurality of plant cells in whole plants or suspensions; and
 - (e) extracting and purifying the human coagulation factor VIII from the plurality of transgenic plant cells.
2. The method as recited in claim 1, wherein transferring is by direct particle bombardment.
3. The method as recited in claim 1, wherein transferring is by agrobacterium mediated transformation.
4. The method as recited in claim 1, wherein transferring is by pollen transformation.
5. The method as recited in claim 3, wherein agrobacterium mediated transformation comprises the steps of:
 - (a) placing the subcloned plant expression vector to an agrobacterium;

(b) co-cultivating the agrobacterium containing the subcloned plant expression vector with the plurality of plant cells.

6. A method of producing a human coagulation factor VIII from plant cells,
5 comprising the steps of:

- (a) introducing an encoding sequence for production of human coagulation factor VIII into a plant expression vector in the plant cells;
- (b) obtaining a positive transformant of the plant cells, the positive transformant carrying genetic material encoding the production of the human coagulation factor VIII;
- (c) cultivating the positive transformant; and
- (d) obtaining the human coagulation factor VIII.

7. The method as recited in claim 6, wherein said encoding sequence is selected from the group consisting of copy DNA, genomic DNA and combinations thereof.

8. The method as recited in claim 6, wherein factor VIII is cultivated in the whole plant.

9. The method as recited in claim 6, wherein factor VIII is cultivated in a plant tissue culture.

10. The method as recited in claim 6, wherein factor VIII is extracted and purified by a process selected from the group consisting of protein precipitation, ultrafiltration, affinity chromatography, and electrophoresis.

11. The method as recited in claim 6, wherein said human coagulation factor VIII is an amino acid sequence substantially that of human coagulation factor VIII.

12. The method as recited in claim 6, wherein said encoding sequence encodes a full length of said human coagulation factor VIII .

13. The method as recited in claim 6, wherein said encoding sequence encodes a full length of said human coagulation factor VIII deleting the B-domain.

14. The method as recited in claim 6, wherein said encoding sequence encodes a full length of human coagulation factor VIIIa .

15. The method as recited in claim 6, wherein said encoding sequence encodes human/porcine factor VIII wherein the A2 epitope of human factor VIII is replaced with the analogous porcine sequence.

16. The method as recited in claim 6, wherein said encoding sequence encodes factor VIII heavy chain protein.

17. The method as recited in claim 6, wherein said encoding sequence encodes factor VIII light chain protein.

18. The method as recited in claim 6, further comprising modifying the encoding sequence by adding a regulatory element selected from the group consisting of leader sequences, signal peptides, transcription promoters or enhancers, and transcription terminators.

5 19. The method as recited in claim 6, wherein said encoding sequence is provided by adding said transcription promoter to the upstream or 5' end of the encoding sequence; and adding said transcription terminator to the downstream or 3' end of the encoding sequence.

20. The method as recited in claim 19, further comprising adding an additional regulatory element encoding a signal peptide, said additional regulatory element added between the transcription promoter and the upstream 5' end of the encoding sequence.

21. The method as recited in claim 20, further comprising adding a regulatory element encoding an untranslated leader sequence between the transcription promoter and the additional
15 regulatory element encoding the signal peptide to enhance mRNA stability.

22. The method as recited in claim 20, further comprising adding a regulatory element encoding an untranslated leader sequence at the downstream or 3' end of the encoding sequence to enhance mRNA stability.

20 23. A method of producing a human coagulation factor VIII using an agrobacterium mediated transformation, comprising:

(a) modifying a coagulation factor VIII encoding sequence for subcloning into a plant expression vector;

- (b) subcloning the encoding sequence into the plant expression vector;
- (c) transferring the plant expression vector to agrobacterium;
- (d) co-cultivating a portion of the transgenic plant cells with the agrobacterium;
- (e) selecting positive transformants from the co-cultivated culture on an antibiotic

5 selective media;

- (f) permitting growth of the transgenic plant cells in whole plants or suspensions; and
- (g) extracting a quantity of human coagulation factor VIII from the plant cells.

ABSTRACT OF THE DISCLOSURE

A method is provided for the production of human coagulation factor VIII, for use in the treatment of human beings diagnosed to be deficient in factor VIII coagulant activity, in both whole plants and plant cell culture. Also provided are expression vehicles for the proper transformation of plant tissue for the production of factor VIII, as well as transformed plant cells and processes for producing human factor VIII using plant molecular biology techniques.

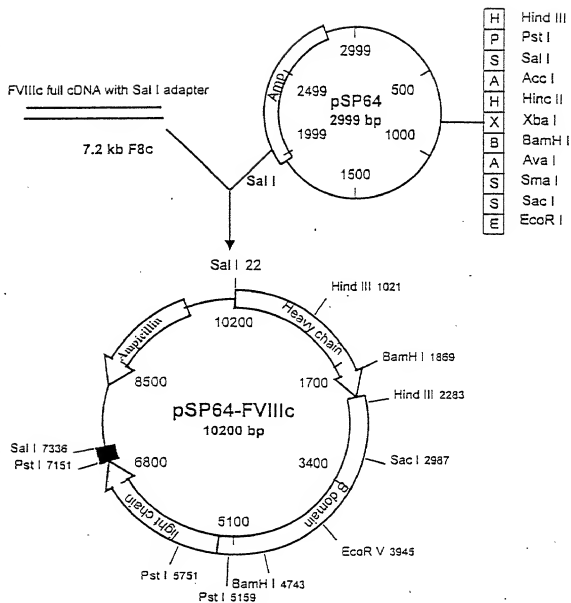


FIGURE 1

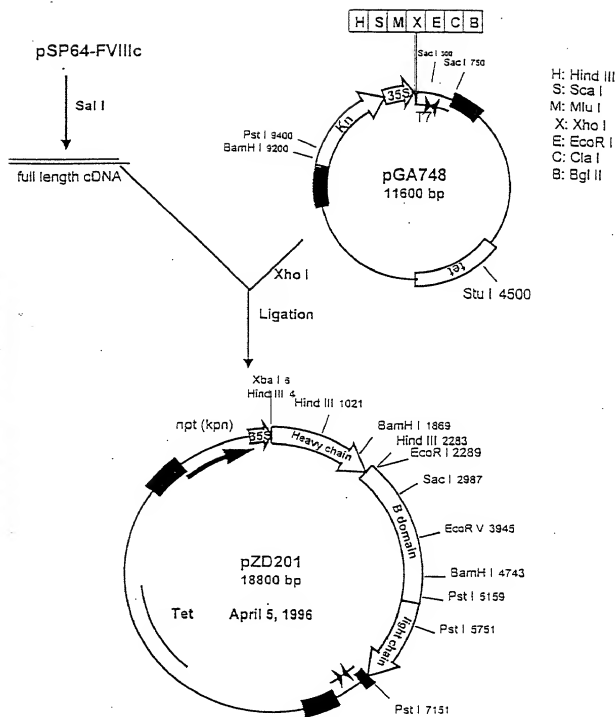


FIGURE 2

S1

S2

SR

1004-3

1006-2

1006-3

FIGURE 3

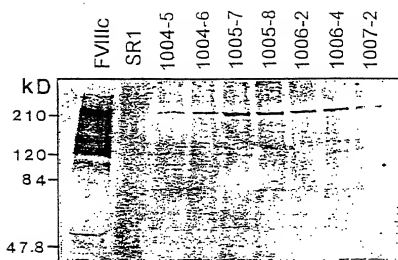


FIG. 4

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**METHOD OF PRODUCING HUMAN COAGULATION FACTOR FROM WHOLE PLANTS OR
PLANT CELL CULTURES**

(Attorney Docket No. 059440/0128)

the specification of which (check one)

☐ is attached hereto.

☒ was filed on June 6, 2000 as United States Application Number _____ and was amended on _____ (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe that the same invention was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I HEREBY CLAIM foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date

I HEREBY CLAIM the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Application Number	Parent Filing Date	Parent Patent Number
09/080,010		5/14/1998	Pending

I HEREBY APPOINT **Stephen R. May, Reg. No. 29,255** of the Battelle Corporation, and the following registered attorneys and agents of the law firm of FOLEY & LARDNER to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith:

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I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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